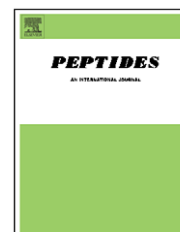


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Transepithelial flux of an allatostatin and analogs across the anterior midgut of *Manduca sexta* larvae in vitro

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ABSTRACT

The transepithelial flux of cydiastatin 4 and analogs across flat sheet preparations of the anterior midgut of larvae of the tobacco hawkmoth moth, *Manduca sexta*, was investigated using a combination of reversed-phase high-performance liquid chromatography (RP-HPLC), enzyme-linked immunosorbent assay (ELISA) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The lumen to hemolymph (L–H) flux of cydiastatin 4 was dose and time-dependent, with a maximum rate of flux of c. 178 pmol/cm²/h measured after a 60-min incubation with 100 µmol/l of peptide in the lumen bathing fluid. The rates of flux, L–H and H–L, across the isolated gut preparations were not significantly different. These data suggest that uptake across the anterior midgut of larval *M. sexta* is via a paracellular route. Cydiastatin 4 was modified to incorporate a hexanoic acid (Hex) moiety at the N-terminus, the N-terminus extended with 5 P residues and/or the substitution of G⁷ with Fmoc-1-amino-cyclopropylcarboxylic acid (Acpc). The incorporation of hexanoic acid enhanced the uptake of these amphiphilic analogs compared to the native peptide. Analogs were also more resistant to enzymes in hemolymph and gut preparations from larval *M. sexta*. A modified N-terminus gave protection against aminopeptidase-like activity and incorporation of Acpc inhibited endopeptidase-like activity. Although analogs were stable in the hemolymph, they were susceptible to amidase-like activity in the gut, which appears to convert the C-terminal amide group to a free carboxylic acid, identified by an increase in 1 mass unit of the peptide analog.

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1. Introduction

The use of orally administered insect neuropeptides for a biorational approach to pest control is considered not to be effective due to their poor absorption characteristics in the alimentary canal and their rapid degradation by gut enzymes. However, a number of studies have demonstrated that peptides and proteins are able to penetrate the gut epithelium of an insect in an active form. The peptides proctolin, trypsin-modulating factors (TMOF) and pheromone biosynthesis-activating peptide (PBAN) have all been detected in the insect

hemolymph and found to be biologically active after oral administration [11–13,33,38]. Proteins, such as albumin and the lectin *Galanthus nivalis* agglutinin (GNA) have also been shown to traverse the insect gut [14,20,31], and the latter has been used as a recombinant fusion protein, comprised of *Manduca sexta* allatostatin attached to GNA, to deliver allatostatin across the insect gut following oral ingestion [21]. Modifications to the primary structure of peptides, using amino acid mimics and fatty acid moieties, have also been undertaken to enhance their stability to enzymes in the gut, and their ability to penetrate the gut epithelium [27–30,34].

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Oral delivery of peptides, or biologically active analogs, to the insect hemolymph could result in an endocrine imbalance and disruption of the physiological processes that peptides regulate as a means of insect pest control, such as feeding, growth and development. Cydiastatin 4, an A-type allatostatin, which has been identified in various Lepidoptera including *M. sexta* [6–8,16,17,19], has been shown to inhibit foregut peristalsis in larvae of the tomato moth, *Lacanobia oleracea* in vitro [8,18,19,24]. This peptide is localized in the frontal ganglion and nerves of the stomatogastric nervous system that innervate the muscles of the gut, suggesting a role in the regulation of feeding activity [19]. Disruption of feeding activity could be a means to control lepidopteran pests; injection of the C-type allatostatin, *M. sexta* allatostatin (Manse-AS), into *L. oleracea* larvae suppresses feeding activity resulting in death [3]. However, before such a strategy can be considered for practical use by oral application, it is first necessary to understand the stability and uptake of an allatostatin in the gut of a larval lepidopteran so that suitable analogs can be designed to enhance the effects of the native peptide.

The focus of this study was to investigate, using in vitro midgut preparations from *M. sexta* larvae, the transepithelial flux of cydiastatin 4 and analogs designed to enhance the stability and ability of this peptide to penetrate the gut epithelium.

2. Materials and methods

2.1. Experimental animals

M. sexta were reared from eggs supplied by Prof. S. Reynolds, University of Bath, using methods described by Yamamoto [36]. Day 2 fifth stadium larvae were used in all experiments, and were anesthetized by submersion in ice-cold water before use. *L. oleracea* was reared as described by Audsley et al. [3]. Sixth stadium larvae were used for gut bioassays.

2.2. Physiological saline

Saline used was based on that described for *M. sexta* by Chamberlin [15] and contained in mmol/l: 5 MgCl₂; 1 CaCl₂; 5.8 KOH; 6 Na₂HPO₄; 7.7 K⁺-citrate; 2.8 Na⁺-succinate; 10 glucose; 180 sucrose; 3.6 alanine; 9.4 glutamine; 12.8 glycine; 9.7 histidine; 5.6 malic acid; 7.4 proline; 8.9 serine; 4.6 threonine; 10 MOPS. The pH of the saline was adjusted to 6.7 with nitric acid.

For foregut peristalsis assays the composition of saline was (mmol/l) 154 NaCl; 2.7 KCl; 1.8 CaCl₂; 22 glucose and 12 hydroxyethylpiperazine ethanesulphonic acid. The pH of the saline was adjusted to 7.2 [24].

2.3. Synthetic allatostatin

Cydiastatin 4 (ARPYSFGL-amide) and analog cydiastatin 4 α (PPPPPARPYSFGL-amide) were custom synthesized at the Advanced Biotechnology Centre, Imperial College, London, UK. Analogs cydiastatin 4 β (PPPPPARPYSF[Acpc]L-amide), cydiastatin 4 γ (Hex-ARPYSF[Acpc]L-amide) and cydiastatin

4 δ (Hex-PPPPPARPYSF[Acpc]L-amide), incorporating Fmoc-1-amino-cyclopropylcarboxylic acid ([Acpc]) and or hexanoic acid (Hex), were synthesized according to previously described procedures [27].

2.4. Midgut tissues for transepithelial flux studies: flat-sheet preparations

Anterior midguts from larval *M. sexta* were carefully dissected free of fat body and tracheal connections, cut at the junction with the foregut and at the position where the attached Malpighian tubules loop back to the posterior midgut (at the first abdominal segment). Each tissue was opened as a flat sheet and mounted into Ussing-type chambers. The tissue was bathed bilaterally in 1 ml physiological saline at 30 °C, and mixed by bubbling with 100% oxygen. The design of the Ussing-type chambers ensured that there were different saline levels at the two sides of the chamber, which could be used to determine whether there was fluid leakage across the gut epithelium due to tissue damage. After two saline changes, cydiastatin 4 or a cydiastatin 4 analog was added to the lumen side of the tissue. The lumen to hemolymph (L–H) flux was measured by taking aliquots of saline from the hemolymph side of the tissues at different time intervals and analyzing by liquid chromatography and enzyme-linked immunosorbent assay (ELISA). To measure the hemolymph to lumen (H–L) flux of cydiastatin 4, peptide was added to the hemolymph side of the midgut, and aliquots of saline were removed from the lumen side of the tissue for analysis.

At the end of each experiment, 1% amaranth, which is not absorbed across gut epithelia, was added to the lumen side of the flat sheet preparations to check for damage to the tissues that would allow leakage of peptide across the tissue [38].

Analysis of HPLC fractions by MALDI-TOF MS was used to confirm transepithelial flux of intact peptide and/or metabolic products.

2.5. High-performance liquid chromatography

Samples were diluted with 0.1% TFA and loaded, via a Rheodyne loop injector, onto a Jupiter C₁₈ 10 μ m 300 Å narrow bore column (250 mm \times 2.1 mm i.d.; Phenomenex, Macclesfield, UK) fitted with a guard column (30 mm \times 2.1 mm i.d.) of similar packing material. The column was eluted with a linear gradient of 10–50% acetonitrile/0.1% TFA, over 40 min at a flow rate of 0.2 ml/min, and elution monitored at 214 nm, using a Beckman 32 Karat chromatographic system (Beckman Coulter (UK) Ltd.), comprising a dual pump programmable solvent module 126 and a UV detector module 166. Fractions (1 min, 0.2 ml) were collected and concentrated to c. 10 μ l by centrifugal evaporation for mass analysis. The elution positions of cydiastatin 4 and analogs were determined so that ELISA and mass analysis could be conducted on the appropriate fractions.

2.6. Mass analysis of allatostatin and degradation products

Mass spectra were acquired on a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Warrington, UK). The

matrix, α -cyano-4-hydroxycinnamic acid (Sigma–Aldrich) was prepared at a concentration of 10 mg/ml in 50% acetonitrile/0.05% (v/v) TFA. Samples (0.5 μ l) were added to the MALDI sample plate followed by 0.5 μ l of matrix, and dried at room temperature. Standards (bradykinin, angiotensin, somatostatin and adrenocorticotrophic hormone; Sigma–Aldrich (UK)) were added adjacent to samples. Spectra represent the resolved monoisotopic masses ($[M + H]^+$) in reflector mode within the mass range m/z 500–2500. Spectra are the accumulation of 5×50 shots.

2.7. ELISA

An indirect ELISA for cydiastatin 4, and each individual analog, was used to measure immunoreactivity in HPLC fractions, corresponding to the elution positions of cydiastatin 4 and analogs, using methods reported by Audsley et al. [2]. Briefly, HPLC fractions and synthetic cydiastatin 4 or analogs were dried onto multi-well plates (Sigma–Aldrich, UK; cat. no. M4034) at 37 °C then incubated overnight at 4 °C with 100 μ l of 0.1 M bicarbonate (coating) buffer (pH 9.6). Plates were washed three times with 150 μ l of 10 mmol/l phosphate buffer/0.1% TWEEN-20 (PBS), blocking solution (150 μ l; 2% non-fat milk in PBS) was added, and the plates were incubated for 90 min at 37 °C. After a further PBS wash (3 \times), 100 μ l of primary antiserum (dilutions 1:3000–1:5000) was added to each well and the plates were incubated for another 90 min at 37 °C. Goat anti-rabbit antiserum conjugated with horseradish peroxidase (100 μ l; 1:3000 dilution in PBS) was added as secondary antibody to each well after PBS washing (3 \times). Plates were then incubated for 40 min at 37 °C. After final PBS washing (3 \times), 100 μ l of substrate solution (25 mg o-phenylenediamine, 20 μ l H_2O_2 in 25 ml citrate buffer, pH 5.0) were added to each well and incubated for 40 min at 37 °C. The reaction was stopped by addition of 50 μ l 1.0N H_2SO_4 to each well and optical density read at 492 nm on a Labsystems Multiskan MCC/340 (Thermo Electron Corporation, Basingstoke, UK). Absorbance was converted to amounts of cydiastatin 4 or analogs using the standard curves.

2.8. Preparation of hemolymph

Hemolymph was collected from a cut in the horn of larval *M. sexta*, into ice-cold polypropylene tubes containing phenylthiocarbamide to inhibit phenyloxidase activity. Hemolymph was sonicated using a MSE soniprep 150 ultrasonic disintegrator (Jencons (Scientific) Ltd., Forest Row, UK), centrifuged at $12,000 \times g$ for 20 min at 4 °C. The supernatant was stored at –70 °C until use, and was diluted fivefold with physiological saline for assays.

2.9. Preparation of midgut lumen contents

The midguts of five fifth stadium *M. sexta* were ligated at the anterior and posterior ends, removed, washed in physiological saline, and then opened to remove the lumen contents. The contents were diluted approximately 10-fold in ice-cold physiological saline, vortexed, then centrifuged at $12,000 \times g$ for 20 min at 4 °C. The supernatant was stored at –70 °C and diluted to a protein concentration of 1 μ g in 100 μ l physiological saline for assay.

2.10. Metabolism of allatostatin

Cydiastatin 4 γ (2.5 nmol), cydiastatin 4 δ (2.5 nmol) or 1 μ l water (control) was added to 100 μ l of diluted hemolymph or diluted extract of midgut contents. The samples were mixed using a vortex stirrer, and the tubes incubated at 30 °C for varying lengths of time. At the end of the incubation period 500 μ l of methanol was added to stop enzyme activity. Samples were then centrifuged at $12,000 \times g$ for 20 min and supernatant diluted 10-fold with 0.1% TFA for separation by RP-HPLC as described above. The peak heights of known amounts of cydiastatin 4 γ and cydiastatin 4 δ were measured using Beckman 32 Karat software and used to calculate the amount of analog from the peak height of peptide in each sample. A reduction in peak height of analog after incubation with hemolymph or gut extract compared to the 0-min treatment was used as a measure of enzymic degradation of these cydiastatin 4 analogs.

2.11. Foregut contractions

Sixth instar *L. oleracea* larvae were starved overnight, anesthetized with CO_2 and cut open along their dorsal surface to one side of the heart from the head to the third abdominal segment. The cuticle was pinned back to expose the foregut and anterior midgut. After rinsing several times with physiological saline, the gut was bathed in 200 μ l of fresh saline at 22 ± 2 °C and the preparation viewed under a dissecting microscope to observe peristaltic contractions of the foregut. A baseline frequency of contractions was established over 2×1 min periods. Control saline was then replaced by saline containing either cydiastatin 4 γ or cydiastatin 4 δ at various doses. Frequency of contractions was again counted over 2×1 min periods, after which the gut was washed several times to remove peptide (analogs) and return the foregut to baseline peristalsis. Another solution of saline + peptide (analog) could then be added and foregut peristalsis observed again.

3. Results

3.1. ELISA for cydiastatin 4 and analogs

Fig. 1 compares the standard curves for the immunoreactivity of cydiastatin 4 and analogs as detected by ELISA. Both cydiastatin 4 and cydiastatin 4 α were immunoreactive to the A-type allatostatin antisera in a linear dose-dependent manner over the range 1×10^{-14} to 5×10^{-13} mol. Other analogs were not as immunoreactive as cydiastatin 4, with detection limits of 7.5×10^{-14} mol (cydiastatin 4 β), 1×10^{-13} mol (cydiastatin 4 δ) and 2×10^{-13} mol (cydiastatin 4 γ). Their standard curves were linear between c. 1×10^{-13} and 1×10^{-11} mol.

3.2. In vitro transepithelial flux of cydiastatin 4 and analogs across the anterior midgut of *M. sexta*

The uptake of cydiastatin 4 across the anterior midgut of *M. sexta* larvae in vitro was highly variable between tissues and

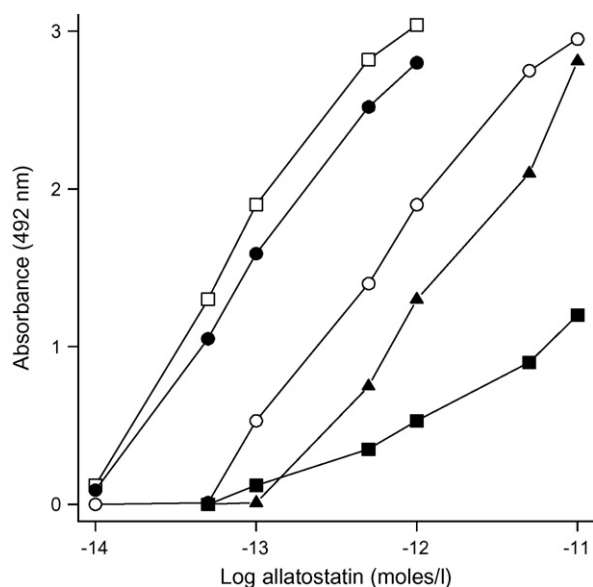


Fig. 1 – Standard curve for the immunoreactivity of cydiastatin 4 (solid circles), cydiastatin 4 α (open squares), cydiastatin 4 β (open circles), cydiastatin 4 γ (solid triangles) and cydiastatin 4 δ (solid squares) to allatostatin antisera.

batches of insects. To limit variability, sets of experiments were conducted on a single batch of insects.

Table 1 shows the L–H flux (measured by ELISA of HPLC fractions) of cydiastatin 4 across the midgut of *M. sexta* larvae over a 60-min period. The transepithelial flux of peptide was concentration-dependent, with no measurable uptake of cydiastatin 4 from the lumen containing 1 $\mu\text{mol/l}$ cydiastatin 4, but intact peptide was detected in the hemolymph saline from higher amounts of cydiastatin 4 in the lumen bathing saline (10, 50 and 100 $\mu\text{mol/l}$).

The time course of flux across the midgut from the lumen saline containing 50 $\mu\text{mol/l}$ cydiastatin 4 to the hemolymph saline is shown in Fig. 2. No measurable peptide was detected after 1 or 5 min, but thereafter the measured amount of cydiastatin 4 in the hemolymph saline increased from 2.2 ± 0.8 pmol after 15 min to 36.5 ± 6.5 pmol after 60 min. The transepithelial (L–H) flux of cydiastatin 4 increased from 45.8 ± 10.6 pmol/cm²/h over the first 15 min to 186.4 ± 33.3 pmol/cm²/h over the next 45 min (Fig. 2). There was no significant difference between the L–H (21.7 ± 2.5 pmol/cm²/h) and H–L (18.6 ± 2.7 pmol/cm²/h) flux of 50 $\mu\text{mol/l}$ cydiastatin 4

Table 1 – Lumen to hemolymph flux of cydiastatin 4 across the anterior midgut of larval *M. sexta* in vitro at different concentrations of peptide in the lumen bathing saline (means \pm S.E., $n = 4$ –8)

Lumen saline cydiastatin 4 concentration ($\mu\text{mol/l}$)	Lumen to hemolymph flux (pmol/cm ² /h)
1	0
10	8.44 ± 2.83
50	21.69 ± 2.49
100	177.78 ± 46.22

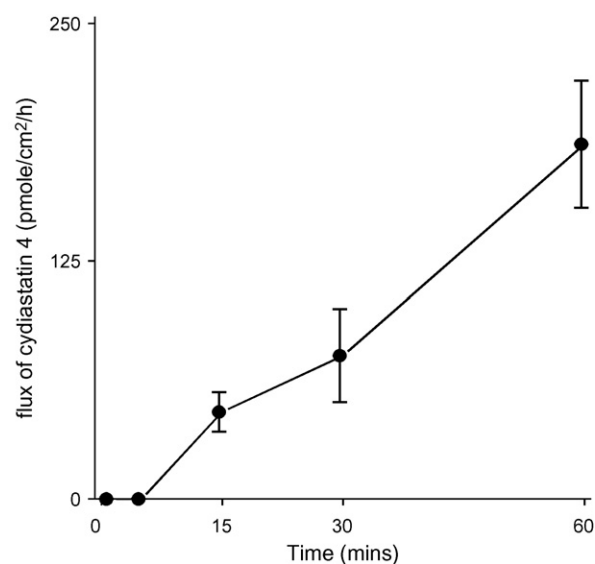


Fig. 2 – The transepithelial flux of cydiastatin 4 across larval *M. sexta* foregut mounted in a modified Ussing chamber over a 60-min period with 50 $\mu\text{mol/l}$ cydiastatin 4 added to the lumen bathing saline (means \pm S.E., $n = 6$ –8).

determined over a 30-min period ($P = 0.45$). The maximum uptake of cydiastatin 4 over a 1-h period was $<1\%$ of the total amount of peptide added to the lumen side of the tissue.

Table 2 compares the transepithelial (L–H) flux of cydiastatin 4 analogs across the anterior midgut of larval *M. sexta* at different concentrations. The L–H flux of cydiastatin 4 α (PPPPARPYSGFGL-amide) is fivefold less than cydiastatin 4 at a concentration of 10 $\mu\text{mol/l}$, but at a higher, 50 $\mu\text{mol/l}$ concentration, there is no significant difference between the fluxes ($P = 0.41$; c.f. Table 1). At lumen concentrations of 1 $\mu\text{mol/l}$, a dose for which no transepithelial flux of cydiastatin 4 could be measured, significant amounts of cydiastatin 4 β (PPPPARPYSF[Acpc]L-amide), cydiastatin 4 γ (Hex-ARPYSGF[Acpc]L-amide) and cydiastatin 4 δ (Hex-PPPPARPYSGF[Acpc]L-amide) could be detected in the hemolymph bathing saline. The L–H fluxes of these peptide analogs were of the same order of magnitude (23–191 pmol/cm²/h) as cydiastatin 4, but were attained using a 10-fold lower dose of peptide.

Table 2 – A comparison between the fluxes of cydiastatin 4 analogs across the anterior midgut of larval *M. sexta* in vitro at different concentrations of peptide in the lumen bathing saline (means \pm S.E., $n = 4$ –8)

Cydiastatin 4 analog	Lumen saline analog concentration ($\mu\text{mol/l}$)	Lumen to hemolymph flux (pmol/cm ² /h)
Cydiastatin 4 α	10	1.6 ± 0.2
Cydiastatin 4 α	50	15.6 ± 4.2
Cydiastatin 4 β	1	23.35 ± 2.7
Cydiastatin 4 β	10	191.1 ± 36.7
Cydiastatin 4 γ	1	63.0 ± 9.3
Cydiastatin 4 δ	1	81.5 ± 21.8

Table 3 – Elution times of cydiastatin 4 and analogs fractionated on a RP-C₁₈ narrow bore column

Peptide	Elution time (min)
Cydiastatin 4	26.2
Cydiastatin 4 α	27.1
Cydiastatin 4 β	27.3
Cydiastatin 4 γ	23.3
Cydiastatin 4 δ	24.2

3.3. Integrity of peptide and analogs

No apparent UV absorbing peaks were measured after HPLC fractionation of saline from the hemolymph side of *M. sexta* midgut mounted in an Ussing-type chamber after various amounts of cydiastatin 4 or analogs had been added to the lumen side of the tissue. However, from mass analysis of fractions corresponding to the elution positions of cydiastatin 4 and analogs (Table 3), mass ions in agreement with synthetic peptides were measured confirming the transepithelial flux of

intact cydiastatin 4 and all four analogs tested. Fig. 3a represents the mass spectra of fraction 27 (26–27 min) from the HPLC separation of hemolymph saline 60 min after 50 $\mu\text{mol/l}$ cydiastatin 4 had been added to the lumen saline. The measured monoisotopic mass (909.5) corresponds to cydiastatin 4, showing the major component in this fraction was the intact peptide. The truncated peptide, cydiastatin 4^{2–8} ($[M + H]^+ = 838.4$) was also present in this fraction. Fig. 3b represents the mass spectra from fraction 28 (27–28 min) of HPLC fractionation of hemolymph saline 60 min after 10 $\mu\text{mol/l}$ cydiastatin 4 β had been added to the lumen saline. The major product in this fraction, transported across the midgut, which had a mono-isotopic mass of 1420.8 in agreement with cydiastatin 4 β , is also the intact peptide. Similarly, in HPLC fractions matching their elution positions, mass ions ($[M + H]^+$) corresponding to cydiastatin 4 α (1394.8), cydiastatin 4 γ (1033.6) and cydiastatin 4 δ (1518.8) were also measured (results not shown). No degradation products were detected in these fractions.

3.4. Stability of cydiastatin 4 γ and cydiastatin 4 δ in hemolymph and the midgut lumen contents from *M. sexta* larvae

When 2.5 nmol of cydiastatin 4 γ or cydiastatin 4 δ were incubated with diluted hemolymph from larval *M. sexta*, no measurable degradation of either peptide analog occurred over a 90-min period (results not shown). However, when the same amounts of analogs were incubated with diluted contents of the midgut lumen from larval *M. sexta*, degradation was rapid. Cydiastatin 4 γ had an estimated half-life of 2.5 min,

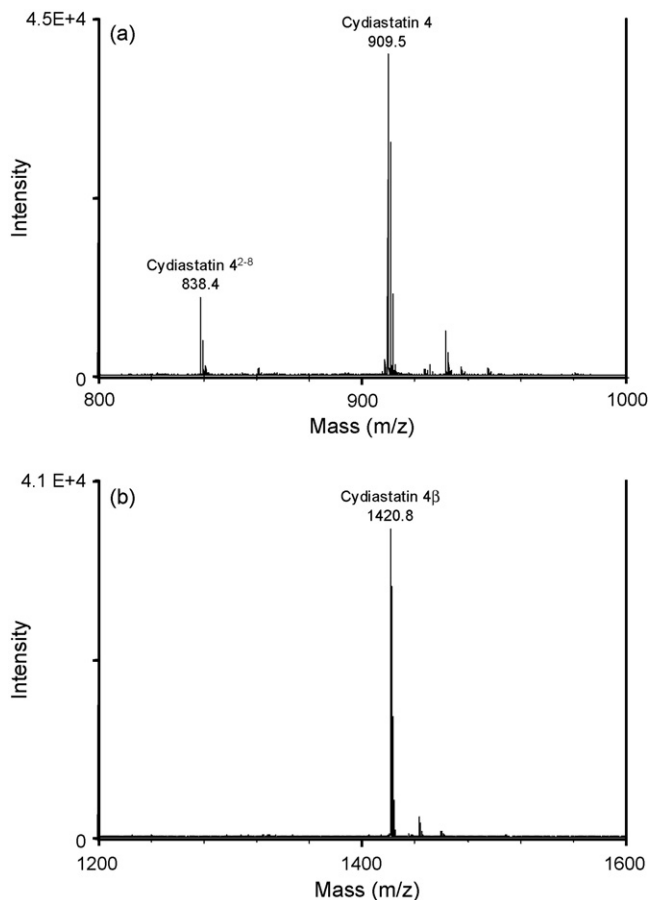


Fig. 3 – (a) Mass spectra of fraction 27 from HPLC separation of hemolymph bathing saline collected 60 min after 50 $\mu\text{mol/l}$ cydiastatin 4 was added to the lumen bathing fluid. (b) Mass spectra of fraction 28 from HPLC separation of hemolymph bathing saline collected 60 min after 10 $\mu\text{mol/l}$ cydiastatin 4 β was added to the lumen bathing fluid. Numerical values represent monoisotopic masses ($[M + H]^+$).

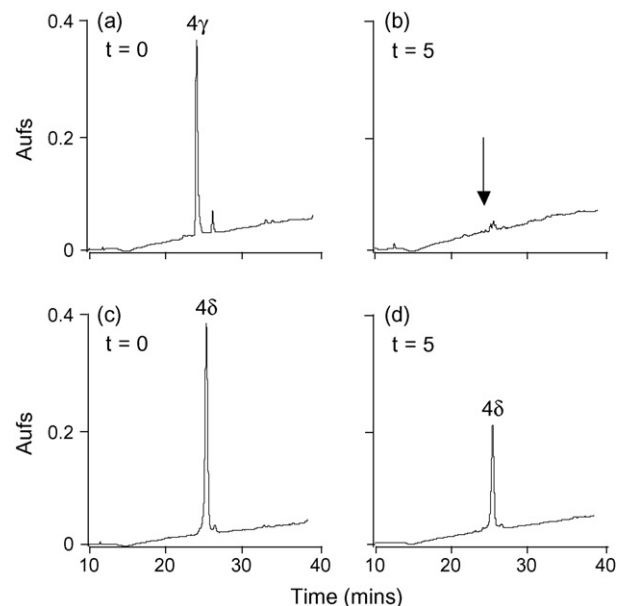


Fig. 4 – Chromatogram of 2.5 nmol cydiastatin 4 γ incubated with *M. sexta* midgut lumen contents, at times 0 (a) and 5 min (b), showing the reduction in the cydiastatin 4 γ peak. The arrow represents the elution position of cydiastatin 4 γ . The chromatogram of cydiastatin 4 δ at time 0 (c) and 5 min (d) after incubation with lumen contents from the midgut of larval *M. sexta*.

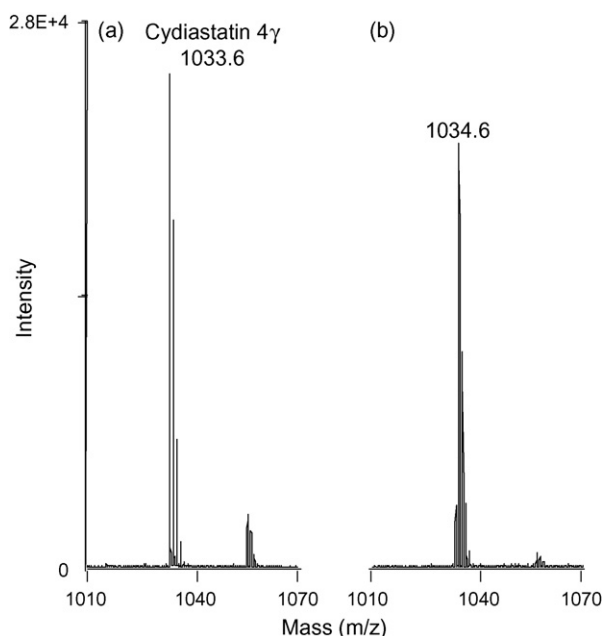


Fig. 5 – Mass spectra of cydiastatin 4 γ (a) and (b) primary product from fractions 24 and 26 of HPLC fractionation of cydiastatin 4 γ + *M. sexta* midgut lumen contents (2-min incubation). Numerical values represent monoisotopic masses ($[M + H]^+$).

the HPLC separation of this analog at times $t = 0$ and $t = 5$ min are shown in Fig. 4a and b. The arrow represents the elution position of cydiastatin 4 γ , showing that most of the intact peptide had been degraded after 5 min with no apparent UV absorbing peaks of the peptide or any metabolic product (Fig. 4b). Mass analysis of HPLC fractions (Fig. 5) identified the

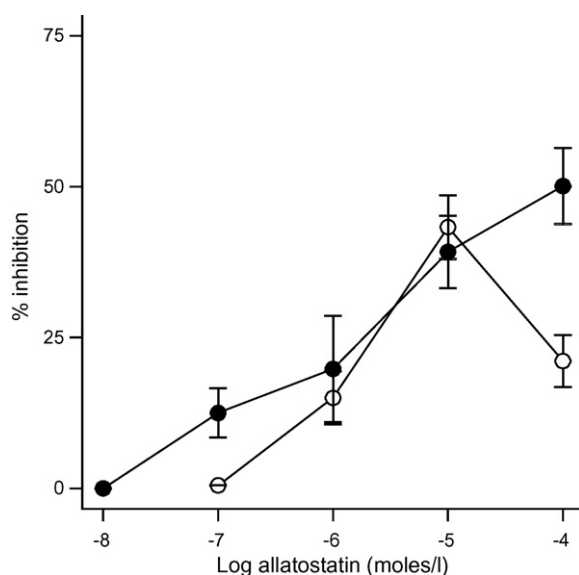


Fig. 6 – Dose-response curves for the inhibition of foregut peristalsis of sixth instar larval *L. oleracea* by cydiastatin 4 γ (solid circles) and cydiastatin 4 δ (open circles), means \pm S.E., $n = 6$ –12.

intact peptide ($[M + H]^+$; 1033.6), in fraction 24 (Fig. 5a) and a mass ion (1034.6) in fraction 26 corresponding to cydiastatin 4 γ plus 1 mass unit (Fig. 5b).

The estimated half-life of cydiastatin 4 δ was c. 4.8 min when incubated with diluted midgut contents. Mass analysis of HPLC fractions identified a mass ion (1518.8) in agreement with cydiastatin 4 δ eluting at 24.2 min, and a mass ion of 1519.8, 1 unit greater than cydiastatin 4 δ , eluting at 26.3 min (mass spectra not shown). No other mass ions corresponding to any metabolic products were identified.

3.5. Effect of cydiastatin 4 analogs on foregut muscle contractions

The inhibition of spontaneous muscle contractions of the foregut of larval *L. oleracea* by cydiastatin 4 γ and cydiastatin 4 δ are shown in Fig. 6. Control rates of foregut muscle contractions were 8.8 ± 0.8 contractions/min, which could be inhibited in a dose-dependent manner by cydiastatin 4 γ or cydiastatin 4 δ by up to 50% over the range 10^{-8} to 10^{-4} M.

4. Discussion

This study clearly demonstrates that, in the absence of soluble enzymes, intact cydiastatin 4 can penetrate the epithelium of the anterior midgut of *M. sexta* larvae, as confirmed by a combination of RP-HPLC, ELISA and MALDI-TOF MS analysis. Some degradation of the peptide occurred during the experimental period, due to tissue-bound or cellular enzymes, resulting in relatively small amounts cydiastatin 4 δ . The L–H flux of this peptide across the anterior midgut was dependent on the peptide concentration in the lumen bathing saline, and intact cydiastatin 4 could be detected in the hemolymph saline when 10, 50 or 100 $\mu\text{mol/l}$ cydiastatin 4 was present in the lumen saline. At lower peptide concentrations in the lumen bathing saline ($\leq 1 \mu\text{mol/l}$) the amounts of cydiastatin 4 penetrating the midgut were below the detection limits of the assay (ELISA) used. The maximum L–H flux of cydiastatin 4 across the anterior midgut of *M. sexta* larvae was $177.8 \pm 46.2 \text{ pmol/cm}^2/\text{h}$, which is $<1\%$ of the amount of peptide (100 $\mu\text{mol/l}$) added to the lumen side of the tissue. By comparison the L–H flux of cydiastatin 4 across the foregut epithelium of larval *M. sexta* was $6560 \pm 830 \text{ pmol/cm}^2/\text{h}$, 2.8% of the total amount of peptide added to the lumen bathing fluid [9], and c. 37 \times greater than the flux across the midgut. The L–H flux of albumin across isolated midgut preparations from *Bombyx mori* larvae, was also dependent on the luminal concentration of the protein and reached a maximum rate of $40 \mu\text{g/cm}^2/3\text{h}$ [14], and approximately 0.55% (0.3 μg) of orally administered [^3H]-proctolin was detected in the hemolymph of *Helicoverpa armigera* larvae over a 2-h period [11]. Assuming similar rates of flux of cydiastatin 4 across the anterior midgut of day 2 fifth instar larvae of *M. sexta* *in vivo*, and a hemolymph volume of c. 800 μl [35], the maximum concentration of peptide in the hemolymph would be 222 nmol/l. The EC_{50} of cydiastatin 4 for the inhibition of foregut peristalsis in larval *L. oleracea* was 0.25 nmol/l [10], although the effects of cydiastatin 4 have not been tested on the foregut of larval *M. sexta*. However, these results would suggest that enough peptide

could penetrate the midgut of this insect to have a physiological effect on the gut. The L–H flux of cydiastatin 4 across the midgut continued to increase over a 1-h period, whereas across the foregut the rates reached a relatively constant level after 5 min [9]. The reason for this difference between the fluxes of cydiastatin 4 across foregut and the midgut is unclear. The H–L flux of cydiastatin 4 across the midgut is also not significantly different to the L–H flux which, together with the L–H flux of cydiastatin 4 being concentration-dependent, supports the movement of this peptide via a paracellular, rather than transcellular, route [14]. There is evidence that some proteins can cross the insect midgut by the paracellular route [23,25] and a similar pathway for small peptides has been described across the intestine of the albino rabbit [37]. Inulin, which has an approximate molecular weight of 5000 Da, can penetrate the midgut of *Schistocerca gregaria* via the paracellular route [38], and hence the midgut is likely to be permeable to smaller molecules such as peptides (e.g. cydiastatin 4). In contrast, albumin appears to cross the insect gut via transcytosis, which is an energy-dependent process [14]. Similarly GNA is absorbed across the gut epithelial cells by transcytosis after binding to carbohydrates on the apical membrane [21]. Proctolin, PBAN and TMOF have all been shown to be absorbed across the insect gut intact and have biological activity *in vivo* although their routes of uptake have not been elucidated [12,13,33,38].

Although cydiastatin 4 can penetrate the midgut in an active form *in vitro*, it is unlikely to do so *in vivo* because it is rapidly degraded by enzymes associated with the gut [5,9,10] and even if successful in traversing the gut would also be susceptible to degradation by circulating enzymes [4,9]. Modifications to a peptide's primary structure to create enzyme-resistant peptide analogs, and enhancing the ability of peptides to penetrate the insect gut epithelium, may increase their effectiveness and potential use in a pest control strategy [26–30,34]. When injected into adult female *Diploptera punctata*, pseudopeptide allatostatin analogs inhibited juvenile hormone biosynthesis and oocyte growth [22]. Peptidase-resistant pyrokinin/PBAN amphiphilic analogs, containing the fatty acid, hexanoic acid, can penetrate isolated, ligated foregut and midgut of *Periplaneta americana* at greater rates than the native peptide [29]. Fatty acid components also enhanced the rates of penetration of pyrokinin neuropeptides through the cuticle of adult *P. americana* and *Heliothis virescens* [28]. Results presented here clearly demonstrate that the L–H fluxes of cydiastatin 4 β , cydiastatin 4 γ and cydiastatin 4 δ are all much greater than the unmodified peptide. The amphiphilic analogs, cydiastatin 4 γ and cydiastatin 4 δ had the greatest rates of flux across the midgut, demonstrating that the increased hydrophobicity due to the incorporation of hexanoic acid at the N-terminus enhances their ability to penetrate the gut wall.

Previous studies on the degradation of cydiastatin 4 by gut and hemolymph enzymes identified the specific amino acids susceptible to hydrolysis [9]. These studies identified two major cleavage sites, the deletion of a single amino acid (A¹) from the N-terminus to produce cydiastatin 4^{2–8}, and cleavage of the C-terminal di-peptide GL-amide to produce cydiastatin 4^{1–6} due to aminopeptidase and endopeptidase activity, respectively [9]. These findings aided in the design of

analogues which are more resistant to enzyme degradation, which included the protection of the N-terminus by adding five proline residues, and replacement of the susceptible G⁷ residue with the amino acid mimic Acpc. Similar substitutions were used to produce an allatostatin analog that was resistant to hemolymph, gut and tissue enzymes in *D. punctata* [27]. Both cydiastatin 4 γ and cydiastatin 4 δ were resistant to hemolymph enzymes over the 90-min incubation period, but were still susceptible to gut enzymes with half-lives of <5 min, similar to that previously reported for cydiastatin 4 [9]. However, degradation does not appear to be due to aminopeptidase(s) and endopeptidases, which have been shown to hydrolyze cydiastatin 4, because the major metabolic product of each analog had a molecular mass of 1 unit greater than the original peptide analog. This increase of 1 mass unit is most likely due to the conversion of the C-terminal amide to the free carboxylic acid by amidase-like enzymes. Similarly, cydiastatin 4 β , which is modified at the N- and C-termini, was resistant to aminopeptidase and carboxypeptidase-like enzymes, but was susceptible to amidase-like activity in the gut, whereas cydiastatin 4 α , which has no C-terminal protection was susceptible to endopeptidase-like activity in the gut and amidase-like enzymes in the hemolymph [10]. The C-terminal amidation appears to be important for biological activity of most insect peptides and hence conversion to the free acid in cydiastatin 4 analogs may have a significant impact on their ability to inhibit foregut peristalsis. The potency of Dippu allatostatin 7 (also an A-type allatostatin) was reduced >10,000-fold when assayed on juvenile hormone synthesis by the corpora allata of *D. punctata* [32], and the free acid form of the insect corticotrophin releasing factor-like diuretic hormone from *M. sexta* is around 1000-fold less potent than the amidated peptide [1].

Modification of cydiastatin 4 to enhance its uptake across the gut *in vitro* and resistance to enzyme degradation has an impact on its biological activity. Analogs cydiastatin 4 α and cydiastatin 4 β , although fully active, are not as potent (apparent EC₅₀ values of 4.2×10^{-9} and 8.3×10^{-7} mol/l, respectively) as cydiastatin 4 (EC₅₀ of 2.5×10^{-10} mol/l) [10]. Results show that cydiastatin 4 γ and 4 δ are more than 100,000 times less potent than cydiastatin 4, and cydiastatin 4 δ also appears to be less active. This loss in potency (and activity) may be due to modifications made in the region of the active site of this molecule, replacing G⁷ with [Acpc]. Clearly, in the design of these cydiastatin 4 analogs there is a trade-off between biological activity and stability and/or ability to penetrate the insect gut.

This study has demonstrated that in the absence of soluble enzymes the insect neuropeptide, cydiastatin 4, can penetrate the anterior midgut of *M. sexta* larvae. This uptake across the gut can be significantly enhanced by the incorporation of the fatty acid, hexanoic acid, at the N-terminus of the peptide. Although modifications to the primary structure of cydiastatin 4 prevented degradation by amino- and endopeptidase-like enzymes, the analogs were still rapidly degraded by other enzymes in the gut, demonstrating that further protection from the hostile environment of the gut is required if this is to be deemed a suitable route of delivery of insecticidal peptide analogs.

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